Enhancing and neutralizing anti-coxsackievirus activities of serum samples from patients prior to development of type 1 diabetes

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Abstract

Studies in prospective cohorts have suggested that enterovirus infections are associated with the appearance of islet autoantibodies that precede later appearance of type 1 diabetes (T1D). It was shown that in addition to an antibody-mediated anti-coxsackievirus (CV)-B neutralizing activity of serum from patients with T1D, there was also enhancing anti-CV-B activity in vitro. In this study the patterns of enhancing and neutralizing anti-CV activities were analyzed from consecutive serum samples collected from children who were followed from birth until they developed T1D in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study and compared to those in non-diabetic control children. The titers of

serum neutralizing activity were analyzed against those CVs which were detected in the stools in these children (CV-B3, CV-B5 or CV-A4) using plaque assay. The enhancing activity of these serum samples was analysed by measuring interferon-alpha (INF-α) production in cultures of peripheral blood mononuclear cells (PBMC) inoculated with a mixture of these viruses and diluted serum. A sustained anti-CV enhancing activity was observed in consecutive serum samples in patients with T1D. The pattern of responses differed between children who developed T1D and control children. In patients, the anti-CV enhancing activity was predominant or even exclusive over the neutralizing activity, whereas in controls the enhancing and neutralizing activities were more balanced or the neutralizing activity was largely predominant. In conclusion, evaluating the anti-enterovirus neutralizing and enhancing activity of serum samples can be useful to investigate further the relationship between enteroviruses and the development of T1D.

Key-words: type 1 diabetes, enterovirus, CV-B3, CV-B5, CV-A4, IFN- α, in vitro, enhancing and neutralizing antibodies.

Introduction

Enteroviruses are non-enveloped positive sense single-stranded RNA viruses belonging to the Picornaviridae family. The Enterovirus genus encompasses thirteen species, out of which viruses present in certain species, namely Enterovirus A [coxsackieviruses A (CV-A) and enterovirus 71] and Enterovirus B [CV-A9, coxsackieviruses B (CV-B1/B6) and echoviruses]

are associated with acute or chronic human diseases including cardiomyopathies and type 1 diabetes (T1D).[1,2] A possible role of enteroviruses in the pathogenic process leading to T1D has been implicated in several studies showing an increased frequency of enterovirus infections and presence of enteroviruses in the pancreas of T1D patients.[3]

In response to enterovirus infections, neutralizing antibodies are produced by the immune system. It has been shown that in addition to an antibody-mediated anti-CV-B neutralizing activity of serum, there is also an anti-CV-B enhancing activity of diluted serum in vitro. Indeed non-neutralizing antibodies have been shown to be able to enhance CV-B4 infection of monocytes and macrophages through interactions between the virus and cell surface receptors (Fc gamma RII and III and CAR) resulting in the production of IFN-α and other inflammatory cytokines by these cells.[4,5,6] This kind of anti-CV-B enhancing activity of serum can worsen CV-B infection, as shown in both *in vitro* and *in vivo* mouse systems.[7,8]

The enhancing activity of serum samples can be assessed by measuring the level of IFN-α in the supernatants of PBMC cultures that have been inoculated with the virus mixed with diluted serum. Our previous studies have shown that production of IFN-α increases significantly when PBMC cultures are inoculated with a mixture of virus and serum from T1D patients when compared to PBMC cultures inoculated with the virus alone.[9,10,11] These results suggest that the enhancing activity of serum (also called antibody-dependent enhancement; ADE) may contribute to the pathogenesis of T1D by increasing the severity of enterovirus infections. [2,12,13] Our previous studies in prospective birth cohorts have

implicated that enterovirus infections are associated with the appearance of islet autoantibodies that precede later appearance of T1D.[14,15,16,17] Hence, the current study aimed at evaluating the patterns of enhancing and neutralizing anti-enterovirus activities in consecutive serum samples collected from children who later developed T1D and to investigate the relationship between the antibody patterns and the development of islet autoantibodies and T1D.

Materials and Methods

Study population

The study subjects included 12 children from the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study born between 1998 and 2001. The DIPP study observes, since 1994, infants from birth who are at risk of T1D based on their HLA DR/DQ genotypes until the age of 15 years or manifestation of T1D (for basic design of DIPP study, see Kupila *et al.*, 2001).[18] Briefly, newborn infants are first screened for genetic risk of T1D HLA-DQB1 alleles and those with risk-associated genotypes are regularly followed for islet autoantibodies including islet cell autoantibodies (ICA) and autoantibodies to insulin (IAA), tyrosine phosphatase-related IA-2 protein (IA-2A) and glutamic acid decarboxylase 65 (GADA). Serum samples have been collected every 3-12 months and stool samples monthly.

We designed a nested case-control study to include six pairs of case-control children with the matching criteria including genetic susceptibility to T1D, date of birth, place of birth, and sex.[19] The six case children had become persistently positive for multiple islet autoantibodies

and eventually five of them have developed T1D by the end of this study. Case and control children were positive for CV-B3, CV-B5, or CV-A4 in stool samples. Case children were positive for CV-B3, CV-B5, or CV-A4 in stool samples before seroconversion at some point during their follow up period. The six control children had no detectable T1D-associated autoantibodies during the follow-up and were pair-wise matched with the case children.

The DIPP study has ethical approval from the hospital districts of Southwest Finland (Turku University Hospital), Pirkanmaa (Tampere University Hospital), and Northern Ostrobothnia (Oulu University Hospital). Parents gave their informed written consent to the participation in the DIPP study.

Serum and stool samples

Serum samples have been collected at each follow-up visit performed at 3- to 12-months intervals and stool samples have been collected every month. Serum samples were not heat-inactivated before assays. The stool samples were analyzed by RT-PCR followed by sequencing of the viral genome for enterovirus detection and identification in stools as explained elsewhere.[20] Type 1 diabetes-associated auto-antibodies were also detected from all serum samples by indirect immunofluorescence or radiobinding assays.[19,21]

Cells

PBMC

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coat pellet provided by the French Blood Center (Lille, France). After a separation over a Ficoll-Plaque™ PLUS solution (GE HealthCare) by centrifugation at 500 g for 30 min at 20 °C, the PBMC fraction was collected and washed twice with RPMI 1640 medium (Fisher Scientific®, IllKirch, France). Cells were re-suspended in RPMI 1640 supplemented with 10% of fetal calf serum (FCS) (Fisher Scientific®) 1% of penicillin and streptomycin (Fisher Scientific®), and 1% of L-glutamine (Fisher Scientific®). They were plated at $5x10^4$ cells/well in 96-well tissue-culture plates (Fisher Scientific®).

RD, Vero and HEp-2 cells

Rhabdomyosarcoma cells (Human) and Vero cells (Monkey, ATCC) were grown in DMEM medium with 10% FCS. HEp-2 cells (BioWhittaker) were grown in Minimal essential medium (MEM) (Fisher Scientific®, IllKirch, France) supplemented with 10% heat-inactivated FCS, 1% L-glutamine, and 1% penicillin and streptomycin. This cell line was used for CV-B3 and CV-B5 culture for virus titration.

Viruses

CV-B3, CV-B5, and CV-A4 strains were obtained from ATCC and were propagated and titrated in HEp-2 (for CV-B3 and CV-B5) and RD cells (for CV-A4). Herpes Simplex Virus (HSV) 1 (VR-260, ATCC®) was propagated and titrated in Vero cells. The titres of virus suspensions

were determined by end point dilution assay using the Reed-Muench method. Aliquots of virus preparations were stored at −80 °C.

Inoculation of viruses into PBMC cultures

The enhancing activity of serum samples was evaluated through the production of IFN-α by PBMCs. The virus suspensions (CV-B3, CV-B5, or CV-A4) mixed with diluted serum from DIPP children (1:1000) were incubated for 1 hour, followed by addition of these mixtures to PBMC cultures (the final Multiplicity of infection (MOI) was 5, that is 2.5 x 10^5 TCID50/well).

PBMC isolated from three buffy coats were used. PBMC from one buffy coat was used for testing the enhancing activity of serum samples against CV-B3, from another one for testing the enhancing activity of serum samples against CV-B5, and from the last one for testing the enhancing activity of serum samples against CV-A4. Controls were (1) PBMC culture inoculated with either diluted serum or virus suspension (CV-B3, CV-B5 or CV-A4) alone and (2) PBMC culture inoculated with virus suspension mixed with diluted human control serum without enhancing activity. The human serum used as negative reference for enhancing activity assay was provided by the Department of Clinical Virology, CHU of Lille. The detection of neutralizing activity against CV-B3, CV-B5 and CV-A4 of this serum was negative.

The cultures were incubated for 24h hours at 37°C in a humidified atmosphere containing 5% CO2. At the end of the incubation period, the supernatants were harvested and stored at -80°C for IFNα detection by ELISA. PBMC cultures inoculated with HSV-1 served as positive controls of IFNα production.[22] For each serum sample the enhancing activity was run in duplicate.

The supernatants of both wells were pooled. The level of IFN-α in the pooled supernatant was the mean value of determination by ELISA in duplicate.

IFN-α

The level of IFNα obtained in the supernatants were determined using the IFNα pan-specific ELISA kit (Mabtech®, Sophia Antipolis, France) that allows the detection of IFN-α subtypes 1/13, 2, 7, 8, 16, and 17. The manufacturer's guidelines were strictly followed for the assay and the detection range was 7-700 pg/mL.

Neutralizing activity assay

The virus seroneutralization assay was used to determine the titers of neutralizing activity of serum samples against the virus detected in the stools of children. A standard plaque reduction assay detected neutralizing antisera. Serial dilutions of sera were incubated with approximately 100 PFU of infectious virus for 1 h at 37°C, continued overnight at room temperature. Neutralizing reactions were then added to 95% confluent GMK cells in 12 well plates (Nunc). After 1 h incubation at 37°C to let virus attach to cellular receptors, a semi-solid media (MEM supplemented with 0.67% carboxymethyl cellulose) was added to cell monolayer and plates were incubated for 2 days at 37°C in a humid chamber with 5% CO_2. Assay was developed by fixing the cells with formaldehyde solution (4%) containing crystal violet to visualize the plaques. The mean number of plaques in 3 control wells was determined. A reduction in plaque number ≥80% in test well compared with untreated virus

suspension was considered positive. If the number of plaques was between the cutoff value +/-2 plaques , the assay was repeated to ensure the accuracy of the results [17].

Statistical analysis

The statistical analyses were performed using the GraphPad Prism® V6.0 software. Comparisons were conducted with Mann–Whitney U test. A p-value 0.05 was considered significant.

RESULTS

Pattern of neutralizing and enhancing anti-enterovirus activities of serum from children

Nine consecutive serum samples were collected from the child #c1 along a follow-up period for 100-months. CV-B3 was identified in a stool sample obtained from this individual at the age of 13 months. An anti-CV-B3 neutralizing activity of serum peaked after the detection of CV-B3 in stools remaining elevated during later follow-up (titre value up to 2048 on month 99) (Fig. 1A c1). Relatively high enhancing activity was detected in four follow-up serum samples (IFN-α levels ranged from 168 to 863 pg/mL) whereas in other samples the values were lower (25 to 87 pg/mL) (Fig.1A c1).

CV-B5 was identified in stools at the age of 27 months in child #b whose eight consecutive serum samples were collected along a follow-up period for 62-months. Anti-CV-B5

neutralizing activity peaked after the infection (ranging from 4 to 256) but a moderate enhancing activity (with a maximum IFN-α value of 144 pg/mL (Fig. 1A c2) was seen already before the infection.

CV-A4 was identified in a stool sample obtained from the four remaining children (#c3 - c6) whose 5 to 9 consecutive serum samples were collected along follow-up periods stretching over 24 to 60 months (Fig. 1A c3 - c6). An anti-CV-A4 neutralizing activity (titre values up to 512) increased in serum samples collected after the CV-A4 infection. There was no neutralizing activity in serum samples collected prior to virus identification in three of these children. However, high anti-CV-A4 neutralizing activity (titre value 512) was observed in a serum sample collected already at the age of one month from one of these children (#c3) (Fig. 1A c3). This child was CV-A4 positive in the stool sample collected at the age of 10 months (Fig. 1A c3). The neutralizing activity of every serum sample was associated with an enhancing activity (2 to 2000 pg/ml IFN-α).

Pattern of neutralizing and enhancing anti-enterovirus activities of serum from children with T1D and/or with T1D auto-antibodies

The pattern of serum neutralizing and enhancing anti-enterovirus (CV-B3, CV-B4, or CV-B5) activities was studied in the six patients and matched controls who all were positive for CV-B3, CV-B5, or CV-A4 in stools (Fig. 1B). Larger inter-individual variations was seen in both

neutralizing and enhancing antibody titres in patients (#a1 - a6) compared to controls (Fig. 1B a1 – a6).

There was no neutralizing activity in serum samples collected prior to virus detection in stools except in two individuals. An anti-CV-B5 neutralizing activity of serum (titre value 1024) was found one month prior to the detection of CV-B5 in stools (on month 13) in one patient (#a2) (see Fig. 1B a2). An anti-CV-A4 neutralizing activity, but at a lower level (titre value 4), was observed in month 18 about one month prior to the detection of CV-A4 in stools in another case child (#a3) (see Fig. 1B a3). Otherwise, a neutralizing serum activity was seen only in samples obtained after the detection of an enterovirus in the stool sample. In the patient with CVB3 in the stool sample (#a1) (Fig. 1 Ba1), the rate of anti-CV-B3 neutralizing activity of serum fluctuated substantially over time (up to 1024 in month 19, then as low as 16 in month 26 and 36 and then up to 1024 in month 48). A major difference between the patients and controls was the predominance of the enhancing serum activity compared with the neutralizing activity (see Fig. 1B). A predominant enhancing activity was observed in most of the consecutive samples obtained from the patient with CV-B3 (#a1), as well as the patient with CVB5 (#a2) along with three out of four patients with CV-A4 (#a3 – a6) (Fig. 1 Ba1 – a6). However, one of the patients with CV-A4 positivity in stools (#a6) had low enhancing antibody activity (15 pg/mL) (Fig. 1B a6). This child had also low neutralizing activity. A predominant anti-CV-A4 enhancing activity was seen in one (in month 26) of his four consecutive samples collected over a follow up period for 16-months following virus identification in the stool sample (Fig. 1 B a6).

T1D-associated autoantibodies were present in the serum sample of each patient following the identification of CVB5 or CV-A4 in the stools sample: (1) ICA and GADA in three patients with CV-A4 (#a3 #a5 and #a6) (Fig. 1 Caa2 and Fig. 1 Caa3, aa5 and aa6), (2) ICA, GADA, and IA2 in the other patient with CV-A4 (#a4) as well as in the patient with CVB5 (#a2) (fig. 1 Caa4 and fig. 1 Caa2). The presence of autoantibodies in the serum sample was associated with an anti-enterovirus activity that was mainly enhancing activity. The sera taken prior to autoantibody appearance had no enhancing/neutralizing activity against the enteroviruses identified in stools in four out of the six patients whereas an anti-CV-A4 activity of serum samples was detected prior to the identification of CV-A4 in the stools sample in one patient (#a5) (see Fig. 1 Ba5 and Fig. 1 Caa5). T1D autoantibodies (ICA) and anti-CV-B3 enhancing activity were present in the serum sample of a patient (#a1) with CV-B3 in the stool sample along time (57 months) prior to the appearance of autoantibodies (see Fig 1 Ba1 and Fig. 1 Caa1). During this period, an anti-CV-B3 activity was detected in two out of eight serum samples.

Five out of six individuals with T1D autoantibodies in their serum developed clinical disease. These patients had a predominant enhancing anti-enterovirus activity in consecutive serum samples collected prior to the presentation of T1D. An anti-CV-B5 predominant enhancing activity in five consecutive serum samples through month 20 to 32 was observed in the patient #a2 who developed the disease on month 43 (Fig. 1 Ba2). An anti-CV-A4 predominant enhancing activity in four consecutive serum samples collected through months 24 to 65 was observed in a patient (#a3) who presented with T1D in month 87, as

presented graphically (Fig. 1 Ba3). An anti-CV-A4 predominant enhancing activity was also seen in seven consecutive serum samples collected through months 23 to 62 and in five consecutive serum samples collected through months 22 to 60 in two patients (#a4 and #a5) who presented with T1D in month 62 and 60 respectively (Fig 1 Ba4 and Ba5). However, an anti-CV-A4 predominant enhancing activity at a lower titre was observed in one out of three consecutive samples (collected through month 19 to 29) in one patient (#a6) who was diagnosed with T1D in month 29 (Fig. 1 Ba6).

The different patterns of values of neutralizing activity and of enhancing activity obtained from serum collected after identification of virus in stool samples have been studied (see figure 2). The mean level of enhancing activity was higher in patient #a3 than in matched control #c3 ($p < 0.05$). The mean level of neutralizing activity in patient #a5 was lower than in controls #c5 ($p < 0.05$). The levels of neutralizing and enhancing activities were lower in patient #a6 than in control #c6 and an enhancing activity against CV-A4 was found in only one serum sample of the patient #a6 ($p < 0.05$) (see figure 2 A and B). The ratio of enhancing and neutralizing activity values in each serum sample was calculated followed by the mean calculation of ratio values for each individual. The mean of these values obtained with serum samples of patients was higher than the one obtained with serum samples of matched controls (18.01 +/-15.16 vs 2.99 +/- 2.76 $p < 0.05$) (figure 2 C).

Discussion

In many respects the current study is different from previous studies carried out to evaluate the possible role of enhancing antibodies in T1D. Most importantly, this is the first prospective study where neutralizing and enhancing anti-enterovirus activities were analyzed from consecutive serum samples collected from children prior to the diagnosis of T1D and covering time periods before and after enterovirus infections that were diagnosed by detecting the virus in stool samples.

Several considerations are noteworthy. The serum samples were obtained from children followed from birth in the Finnish T1D Prediction and Prevention (DIPP) study covering different stages of the beta-cell damaging process including the start of islet autoimmunity (appearance of islet autoantibodies). Both case and control children carried HLA risk genes for T1D and were additionally carefully matched for possible other confounding factors including date of birth, sex and geographic region. Such prospective collection of biological samples and data is the most suitable and powerful approach for analyzing and evaluating the relationship between factors associated with a disease. The available volume of stored serum samples from such individuals is usually limited; especially from infants. A small volume of serum was required to investigate the neutralizing activity and for testing the enhancing activity against the identified serotypes (9 µL and 12 µL respectively). In order to analyze the intra-individual variations of serum activity, the same batch of buffy coat derived PBMCs was used for testing serum samples from the control and paired patient, and all the consecutive serum samples for case-control pairs were tested in the same run.

In our previous studies anti-CV-B4 and anti-CV-B3 enhancing activity was analyzed from serum samples collected from controls and patients with established T1D.[9,10,11] In the present study, we found that serum enhancing activity against CV-B5 and CV-A4 in vitro was elevated already prior to the diagnosis of T1D, as case children had a higher IFN-α response to virus-serum mixture compared to control children.

For the first time, we were able to standardize this case-control comparison by analyzing enhancing activity before and after the infection in case and control children who were infected by the same enterovirus types. The virus was not isolated from the stools samples of the individuals, but the identification of the genotype by nucleotide sequencing opened the possibility to study the activity of serum against the relevant serotype of ATCC reference strains.

The neutralization assay provides a reflection of the activity of biological samples due to neutralizing antibodies against the challenging virus.[23,24] A neutralizing activity is observed soon after the infection and the responses are usually long-lasting and serotype-specific. This was also seen in the current study. However, in some children the neutralizing antibodies were detected prior to virus detection in stool sample, which is most likely explained by long shedding of the virus into stools and lack of any stool sample that would have been collected in the acute phase of the viral disease (stool samples were collected according to a predetermined monthly schedule).

Thus, it can be assumed that the observed neutralizing activity is relevant and is induced by the serotype identified in the stool samples. Nevertheless, this activity may be the result of reactivation of the immune response to the infection as suggested by the detection of a neutralizing activity of a serum sample collected 10 months prior to the serotype identification in the stool samples of two control subjects (one was CV-B3 positive and the other CV-A4 positive). Furthermore, it cannot be excluded that in these individuals the neutralizing serum activity observed prior to the serotype identification was due to heterotypic reactions in response to infections with other unidentified serotypes[25].

As far as individuals with CV-A4 infections were concerned, there was an anti-CV-A4 enhancing activity of serum samples collected 10 months prior to the presence of this serotype in the stool samples whereas there was no neutralizing activity against this virus in these samples (see fig 1A c4). It is possible that this is due to cross-reacting non-neutralizing enhancing antibodies. However, in previous studies we found that enhancing antibodies were not cross-reactive between serotypes but rather induced by the homotypic virus. [10] Furthermore, the neutralizing and /or enhancing activities of serum samples obtained a few months after birth (3 months) before the detection of virus in stools, as observed in some individuals (controls # c1 – c4 and patient #a5), can be due to maternal antibodies.

The enhancing activity of serum samples from patients obtained after the detection of virus in stools was predominant except in the case of patient #a6. The pattern of values of neutralizing and enhancing activities of serum samples from this patient was different

compared to those of other patients. An anti-CV-A4 enhancing activity was observed in one serum sample only. The discrepancy between the pattern of results obtained with serum samples from this patient and those of others may be explained by the fact that CV-A4 was possibly not the relevant virus as far as this patient is concerned.

Whether the sustained anti-enterovirus enhancing activity of consecutive serum samples in patients with T1D auto-antibodies reflects a disturbance of the immune system or an odd response to enteroviruses is an open question[26]. The fact that the serum samples from patients mixed with enteroviruses enhanced the virus-induced production of IFN-α by PBMCs in vitro is in line with previous studies suggesting that IFN-α may play a role in pathogenesis of T1D.[2,27,28] Whether the anti-enterovirus enhancing activity observed in at risk subjects or in patients with T1D can be involved in the development of autoimmunity should be explored further.

In conclusion, the anti-enterovirus neutralizing and enhancing activity of consecutive serum samples from controls and patients were investigated in this proof-of-concept study. The number of patients was limited, but the results indicated that the pattern of response to enteroviruses is different in individuals who develop T1D compared to controls. The determination of the pattern of response to enteroviruses as described here could be implemented in future studies based on larger cohorts of patients, at risk individuals and control subjects. Bioassays aimed at evaluating the anti-enterovirus neutralizing and

enhancing activity of serum samples can be useful to investigate further the relationship between enteroviruses and T1D.

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Legend of figure 1: Neutralizing and enhancing anti-coxsackievirus activities of serum samples obtained from controls and from patients prior to the onset of type 1 diabetes.

Consecutive serum samples were obtained from six patients and six matched controls during a follow-up period ranging from birth to the age of 27 to 99 months (x-axis). Neutralizing (black bars) and enhancing (white bars) activities of serum samples are shown for controls (A) and patients (B) against a relevant reference strain of coxsackievirus types that was found in stools of these children by RT-PCR and identified by nucleotide sequencing. The date of sample positive for the detection of virus in stools is indicated by vertical arrows and the type of virus is indicated (CVB3, CVB5 or CVA4). The pattern of T1D-associated autoantibodies (ICA, IAA, IA-2A and GADA) in each patient during the follow-up is shown (C). The date of diagnosis of T1D is marked in the graphs of panel B. In the patient #a3 represented by graph Ba3 T1D was diagnosed at the age of 87 months as marked on the right side of the graph.

Legend of figure 2: Neutralizing and enhancing anti-coxsackievirus activities of serum samples obtained from controls and from patients after the detection of viruses in stools.

Neutralizing (A) and enhancing (B) anti-coxsackievirus activities of consecutive serum samples obtained from six patients (#a1-a6) and six matched controls (#c1-c6) after detection of viruses in stools. The geometric means and the error bars representing the lower and upper 95% CI (A) and the arithmetic means +/- SD (B) are shown. The ratio of

enhancing and neutralizing activity values in each serum sample obtained from the six patients (#a1-a6) and from the six controls (#c1-c6) was calculated followed by the mean calculation of ratio values for each individual, the mean value for a patient and matched control are represented by the same symbol (C). * p value < 0.05.